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Scientific Officer
Combat Casualty Care
Commander Yaffe
Naval Medical Research & Development Command
8901 Wisconsin Avenue
Building 1, Tower 11
Bethesda, MD 20889-5606

Dear Commander Yaffe:

Please find enclosed the final report for the grant (N00014-94-1-0402) entitled "Effects of Lyophilization on Metabolic Integrity of Red Blood Cells."

Sincerely yours,

John F. Carpenter, Ph.D.
Assistant Professor of Pharmaceutics

FINAL REPORT

PRINCIPAL INVESTIGATOR: John F. Carpenter, Ph.D.

INSTITUTION: School of Pharmacy
University of Colorado Health Sciences Center

GRANT TITLE: Effects of Lyophilization on Metabolic Integrity of Red Blood Cells

AWARD AND REPORTING PERIOD: 1 March 1994 - 29 February 1996

OBJECTIVE: The objective was to investigate the integrity of energy metabolism in human red blood cells that had been lyophilized and rehydrated. This work was in collaboration with Dr. Barry Spargo (NRL), who prepared the lyophilized red cells. Retention of adequate energy metabolism (i.e., glycolysis) is essential for the survival and oxygen delivery function of red blood cells.

APPROACH: Energy metabolism was assessed by determining: 1) the levels of AMP, ADP and ATP; 2) the level of 2,3-diphosphoglycerate; 3) and the rate of metabolic heat generation (using isothermal microcalorimetry), which is indicative of the glycolytic flux rate. Lyophilized cells were studied as a function of time after rehydration, washing and equilibration with a physiological buffer at 37°C. For control values, we used fresh red cells (3 days after draw), which were prepared in CPDA-Adsol. Metabolite levels were determined for cells in the initial storage solution. These values and metabolic heat generation rates were determined as a function of time after washing and equilibration with physiological buffer at 37°C. In addition, we used infrared spectroscopy to determine the secondary structure of hemoglobin in fresh, lyophilized and rehydrated cells. Finally, we manipulated metabolism, with reducing agents in the solutions, of the red cells in an attempt to reduce the levels of methemoglobin.

ACCOMPLISHMENTS: First, we had to ascertain what were the appropriate controls against which to compare metabolism of lyophilized/rehydrated cells. The question arose as to whether using freshly drawn cells would provide too stringent criteria, since it was known that liquid-stored cells, which are routinely transfused, would experience depressed metabolic function during prolonged (but FDA-approved) periods of storage in the refrigerator. We found that during storage in CPDA-Adsol, as shown previously, the levels of ATP and 2,3-DPG declined. With up to four weeks of storage, metabolism (i.e., metabolite levels and heat generation rates) recovered to that seen in freshly drawn cells, if the cells were washed and equilibrated with physiological buffer at 37°C. However, during subsequent storage out to the FDA-approved expiration date of 6 weeks, metabolite reduction in cells monitored directly in CPDA-Adsol, were not reversible by this treatment, which simulates *in vivo* conditions. Earlier research by Valerie indicated that such irreversible damage was due to depletion of metabolic intermediates.

To determine the consequences of metabolic dysfunction on *in vivo* survival, we collaborated with Lt. Col. Michael Fitzpatrick (Walter Reed) to study rabbit red blood cells stored in the refrigerator in CPDA-Adsol. There were irreversible lesions in energy metabolism after 4-6 weeks of storage, which correlated with greatly reduced *in vivo* survival of the same cells after transfusion back into the donor rabbit.

Our assessment of lyophilized/rehydrated cells indicated that cells, which could be recovered after washing, had energy metabolism that was almost equivalent to that of freshly drawn cells. However, the methemoglobin levels were elevated. Two possible nonexclusive mechanisms for this elevation were: 1) lyophilization caused unfolding of hemoglobin, which fosters heme oxidation; and 2) the reducing environment needed by cellular

metabolism to convert methemoglobin back to hemoglobin was not optimal. We used resolution-enhanced Fourier transform infrared spectroscopy to study hemoglobin secondary structure directly in the red cells. We found that the protein was unfolded in the dried solid, but refolded when the cells were rehydrated. Such transient unfolding may contribute to the oxidation of the heme group, which we have found to be the case with purified hemoglobin. Thus, it appears to be important to develop formulations that not only lead to recovery of intact cells, but also prevent hemoglobin unfolding during lyophilization.

We also found that incubation of rehydrated cells in physiological buffer at 37°C containing reducing agents (glutathione and NADH) lowered the levels of methemoglobin. However, it took several hours for the maximum effect to be manifested. Dr. Spargo's lab used our reducing agent formulations with their lyophilization buffer and prepared lyophilized cells. The presence of reducing agents did not alter the level of methemoglobin relative to cells lyophilized without this treatment.

RELATED WORK: We showed that antifreeze protein Types I, II and III and several mutants of Type III could inhibit ice recrystallization damage to red cells cryopreserved in hydroxyethyl starch. This activity correlated directly with thermal hysteresis activity. To enable us to reliably quantitate structural differences in proteins from different samples, we developed a new, superior method for determining the degree of similarity between protein infrared spectra. Also, we developed a method (using lactoglobulins as initial model proteins) employing infrared spectroscopy and H-D exchange to "magnify" structural differences between very similar proteins or protein samples. In addition, we determined that polymers protect a multimeric protein (lactate dehydrogenase) during freeze-drying by inhibiting dissociation in the frozen state. Finally, we documented, for the first time, that a protein stabilizer and a denaturant can counteract each other's effects during freezing and drying. The relevant publications are listed below.

PUBLICATIONS:

Dong, A., S.J. Prestrelski, S.D. Allison and J.F. Carpenter, 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. *Journal of Pharmaceutical Sciences* 84: 415-424.

Dong, A., J. Matsuura, S.D. Allison, E. Chrisman, M.C. Manning and J.F. Carpenter, 1996. Infrared and circular dichroism spectroscopic characterization of structural differences between ß-lactoglobulins A and B. *Biochemistry* 35:1450-1457.

Kendrick, B., A. Dong, S.D. Allison, M.C. Manning and J.F. Carpenter, 1996. Quantitation of area of overlap between second derivative amide I infrared spectra to determine structural similarity of a protein in different states. *Journal of Pharmaceutical Sciences* 85:155-158.

Carpenter, J.F. and B. Chang, 1996. Lyophilization of protein pharmaceuticals. In: *Biotechnology and Biopharmaceutical Manufacturing, Processing and Preservation*, K. Avis and V. Wu (Eds.), Interpharm Press, Buffalo Grove, IL, pp. 199-265.

Chao, H., P.L. Davies and J.F. Carpenter, 1996. Effects of antifreeze proteins on red blood cell survival during cryopreservation. *Journal of Experimental Biology* (In Press).

Anchordoquy, T.J. and J.F. Carpenter, 1996. Polymers protect lactate dehydrogenase during freeze-drying by inhibiting dissociation in the frozen state. *Archives of Biochemistry and Biophysics* (In Press).

Allison, S.D., A. Dong and J.F. Carpenter, 1996. Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying and rehydration. *Biophysical Journal* (In Press).

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Heller, M., J.F. Carpenter and T.E. Randolph, 1996. The effects of polymer/protein phase separation on hemoglobin structure in freeze-dried solids. *Journal of Pharmaceutical Sciences* (In Press).

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